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Originals

Phylogenetic Relationships among Subgroups in *Bupleurum falcatum* L. sensu lato (Umbelliferae) Based on Restriction Site Variation of Chloroplast DNA

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Chloroplast DNA restriction site variation analysis was carried out on seven subgroups in *Bupleurum falcatum* L. sensu lato, together with five other *Bupleurum* species. *Bupleurum falcatum* is widely distributed from Europe, Central and East Asia to Japan. Eighty-three site mutations were detected within 65 plants and 29 types of cpDNA were recognized. Phylogenetic analysis resulted in 36 most parsimonious trees. The strict consensus tree indicated a monophyletic clade composed of six subgroups of Far Eastern *B. falcatum* s. l. and *B. longiradiatum* Turcz. This clade excludes the European *B. falcatum* sensu stricto. This result suggests that the Far Eastern *B. falcatum* s. l. is distinguished from European *B. falcatum* s. str. Additionally, the tree showed that within the Far Eastern group, Japanese *B. falcatum* s. l. with the cytotypes 2n = 26 and 32 were clustered into a single clade (clade I) with Chinese *B. falcatum* s. l. with 2n = 26 and *B. komarovianum* Lincz. Chinese *B. scorzonerifolium* Willd. formed the other clade (clade II). Chinese *B. chinense* DC. were polyphyletically included in both clades. These results suggest that Japanese *B. falcatum* s. l. with 2n = 26 and 32, and Chinese *B. falcatum* s. l. with 2n = 26, are not included in the same taxon as Chinese *B. scorzonerifolium* Willd. and differ from *B. falcatum* s. l. with 2n = 20 in Japan, China and Korea.

Key words: *Bupleurum falcatum*, chloroplast DNA, molecular phylogeny, restriction site variation.

Bupleurum falcatum L. sensu lato (Umbelliferae) is a perennial herbaceous species, widely distributed from Europe to Japan. It is well known that the taxonomic treatments of this species differ by investigators, because much variations exist in its external morphology (Wolff 1910, Nakai 1937, Kitagawa 1947, Hiroe 1958, Hara 1954, Ohwi 1965, Tutin 1968, Shan and Li 1974, Li and Sheh 1979, Ohba 1999 and Yamazaki 2001).

Shan and Li (1974), and Li and Sheh (1979) saw that Chinese *B. falcatum* sensu lato as different from European *B. falcatum* sensu stricto. They divided it into about 20 species such as *B. scorzonerifolium* Willd., *B. chinense* DC. and *B. komarovianum* Lincz.

There are four main taxonomic treatments for Japanese *B. falcatum* s. l.; i. e., (1) the same species as European *B. falcatum* s. str. (Wolff 1910, Hara 1954, Hiroe 1958, Ohwi 1965, Ohba 1999), (2) the same species as Chinese *B. scorzonerifolium* (Nakai 1937), (3) an endemic, *B. stenophyllum* (Kitagawa 1947) and (4) the same species as Chinese *B. scorzonerifolium* and *B. chinense*, each of which is distributed in different regions of Japan (Yamazaki 2001).

Ohta (1986, 1991) carried out a cytogenetical study on plants growing in Japan and abroad. He reported that the Japanese plants with three cytotypes, $2n = 20$, 26 and 32, were distributed in distinct regions from each other. Furthermore, they had differing karyological and morphological characteristics from each other, and from those of Europe and China including phyllotaxis, shape of basal leaves and adventitious root (Ohta 1991).

Dried roots ("Saiko" in Japanese) of East Asian *B. falcatum* s. l. are used for "Kampo" (traditional Japanese herbal medicines) as crude extracts in consistent mixture with other herbaceous materials. The consumption of "Saiko" has increased siginificantly since "Kampo" medicine had been produced in factories. According to the "Kampo" clinical description, the "Saiko" derived from Japanese plants with $2n = 26$ has a different quality as compared with that of China (Ohtsuka et al. 1954).

However, in spite of medicinal importance of Japanese plants, their current taxonomy remains uncertain. Accordingly, it is necessary to clarify the relationships among plants of this species which grow in Japan, China and Europe, usuing informative techniques.

In recent years, chloroplast DNA (cpDNA) is increasingly being used in plant systematic studies, and has contributed to solving many systematic problems (reviewed in Crawford 1990, Clegg and Zurawski 1992, Dowling et al. 1996). Among several methods using cpDNA, the restriction site variation analysis has become one of the most useful approaches to reconstruct the phylogeny among inter- and intra-specific taxa.

In this study, we used cpDNA restriction site variation analysis to clarify the phylogenetic relationships of Japanese plants to Chinese and European plants of *B. falcatum* s. l.

Materials and Methods

Plant material——We used accesions of 65 morphologically and karyologically verified 65 plants including seven subgroups of *Bupleurum falcatum* L. sensu lato: (1) Japanese plants with $2n = 20$, (2) Japanese plants with $2n = 26$, (3) Japanese plants with $2n = 32$, (4) Chinese *B. scorzonerifolium* Willd., (5) Chinese *B. chinense* DC., (6) Chinese *B. komarovianum* Lincz. and (7) European *B. falcatum* sensu stricto (Table1). Japanese plants were morphologically verified as *B. falcatum* according to the classification by Ohwi (1965). However, we treated Japanese plants as *B. falcatum* sensu lato in this study because its taxonomic treatment differed among investigators. The names of Chinese plants were based on the classification by Shan and Li (1974), and Li and Sheh (1979). For cytotypes of $2n = 20$ and 26, the plants growing in Northeastern China (Jang et al. 1994, Li et al. 1994, Pan et al. 1995, Ohta unpublished) were also analyzed. In addition, we analyzed several plants growing in different localities within each subgroup to enhance our confidence in inferences about the relationships among subgroups. *Bupleurum* species representing all five sections of

Table 1. Sources and cpDNA types of *Bupleurum* plants used in this study. Subgroup numbers and cpDNA types are assigned in this study. Cytotypes have been investigated by Ohta (1991, unpublished). Classification of seven subgroups is described in the text

Taxon	Subgroup no.	Locality	No. of plants examined	Plant abbreviation	Cytotype (2n)	cpDNA type
JAPAN						
B. falcatum L. sensu lato	1	Izuhara, Nagasaki Pref.	1	JP-F-Na	20	1
	2	Minamiizu, Shizuoka Pref.	1	JP-F-Sh	26	2
	2	Kugino, Kumamoto Pref.	1	JP-F-Ku	26	3
	2	Cult. at Kochi Pref.	1	JP-F-Koc	26	3
	2	Cult. at Tsumura & Co.	1	JP-F-Ts	26	3
	3	Mikazuki, Hyogo Pref.	2	JP-F-Mi1, 2	32	3
	3	Kobe, Hyogo Pref.	2	JP-F-Ko1, 2	32	2, 3
	3	Tojo, Hyogo Pref.	1	JP-F-To	32	3
	3	Saijo, Hiroshima Pref.	1	JP-F-Sa	32	3
B. longiradiatum Turcz.		Higashiyama, Iwate Pref.	3	JP-L-Iw1-3		4
		Okutama, Tokyo Pref.	1	JP-L-To		1
		Kawakami, Nagano Pref.	1	JP-L-Na		1
CHINA						
B. chinense DC.	4	Wanli, Beijing	8	CH-C-Wa1-8	12	5–10
	4	Chongli, Hebei Prov.	1	CH-C-Ch	12	7
	4	Cult. at Hebei Prov.	1	CH-C-He	12	11
	4	Tongshan, Jiangsu Prov.	1	CH-C-To	12	12
	4	Lianyungang, Jiangsu Prov.	1	CH-C-Li	12	1
	4	Wotaishan, Shanxi Prov.	2	CH-C-Wo1, 2	12	13, 14
	4	Shenjang, Liaoning Prov.	4	CH-C-She1–4	12	1
	4	Shanghai Bot. Garden	2	CH-C-Sha1	24*	1
B. scorzonerifolium Willd.	5	Wanli, Beijing	1	CH-S-Wa	12	15
	5	Dongning, Heilongjiang Prov.	1	CH-S-Do	12	15
	5	Daqing, Heilongjiang Prov.	1	CH-S-Da	12	16
	5	Jurong, Jiangsu Prov.	2	CH-S-Ju1, 2	12	17
	5	Nanjing, Jiangsu Prov.	3	CH-S-Na1-3	12	15
	5	Lianyungang, Jiangsu Prov.	1	CH-S-Li	12	15
	5	Liyang, Jiangsu Prov.	2	CH-S-Liy1, 2	12	15, 18
	5	Chuzhou, Anwei Prov.	1	CH-S-Ch	12	19
B. falcatum L. sensu lato	1	Dazu, Sichuan Prov.	1	CH-F-Da	20	1
	1	Donghe, Ningan, Heilongjiang Prov.	1	CH-F-Do	20	1
	1	Pubu, Ningan, Heilongjiang Prov.	1	CH-F-Pu	20	
	2	Luobei, Heilongjiang Prov.	2	CH-F-Lu1, 2	20	3, 22
	2	Shuangyashan, Heilongjiang Prov.	1	CH-F-Sh	26	2
	2	Bawuwunongchang, Mishan, Heilongjiang Prov.	1	CH-F-Ba	26	21
B. komarovianum Lincz.	6	Xiaobeihulinchang, Ningan, Heilongjiang Prov.	1	CH-K-Xi	8	3
	6	Dongfa, Mishan, Heilongjiang Prov.	1	CH-K-Do	8	3
B. longiradiatum Turcz.		Xiaobeihulinchang, Ningan, Heilongjiang Prov.	1	CH-L-Xi	23	
KOREA						
B. falcatum L. sensu lato	1	Cult. at Tsumura & Co.	1	KO-F	20	24
TIBET						
B. candollii Wall. ex DC.		Cult. at Tsumura & Co.	1	TI	25	
EUROPE						
B. falcatum L. sensu stricto	7	Bot. Gard. of Neuchatel, Switzerland	1	EU-F-Ne	16	26
	7	Bot. Gard. of Nat. Hist., France	1	EU-F-Na	16	26
B. rotundifolium L.		Royal Bot. Gard., Kew, UK	1	EU-R		27
B. stellatum L.		Bot. Gard. Berlin-Dahlem, Germany	1	EU-S		2
Outgroup						
B. fruticosum L.		Cult. at Tsumura & Co.	1	EU-Fr		29

*Presumable tetraploid of *B. chinense* (Ohta 1991).

the genus, were also included in order to determine the relationships with *B. falcatum* s. l. (sect. Perfoliata, *B. rotundifolium*, sect. Longifolia, *B. longiradiatum*, sect. Reticulata, *B. stellatum*, sect. Eubupleura, *B. falcatum* and *B. candollii*, and sect. Coriacea: *B. fruticosum*; Wolff 1910).

Bupleurum fruticosum L. is unique in the genus as the sole woody species. In addition, Kondo et al. (1996) reported that this species was placed outside of the Far Eastern *Bupleurum* clade with high probability based on analysis of *rbc*L gene sequences. They treated Japanese *B. falcatum* s. l. as *B. scorzonerifolium* Willd. var. *stenophyllum* Nakai. For these reasons, *B. fruticosum* was treated as an outgroup in this study.

The wild plants and those grown from the wild-collected and cultivated seeds were nursed, and the voucher specimens were deposited in the herbarium of Tsumura & Co.

DNA extraction——Total DNAs were isolated from the fresh leaves (1–3 g) of each plant according to the CTAB method of Doyle and Doyle (1987). For the isolation of sufficiently digestible DNA by restriction enzymes, DNAs were further purified by equilibrium ultracentrifugation in CsCl ethidium bromide gradients (Sambrook et al. 1989). Purity and quantity of the DNAs were estimated by UV absorption at 230, 260 and 280 nm.

Southern blot analysis——Total DNAs (0.3 μg) were digested with 24 restriction endonucleases: *Alw* 44I, *Apa* I, *Ase* I, *Ava* I, *Bam* HI, *Ban* I, *Bcl* I, *Bgl* I, *Bgl* II, *Csp* 45I, *Dra* I, *Eco* O109I, *Eco* RI, *Eco* RV, *Eco* T22I, *Eco* T38I, *Hinc* II, *Hind* III, *Mlu* I, *Sac* I, *Sal* I, *Sca* I, *Xba* I and *Xho* I. The digested DNAs were size-fractionated electrophoretically in a 1.0% agarose gel. The separated restriction fragments were denatured, and transferred to a charged nylon membrane (Hybond-N+: Amersham) by capillary blotting technique (Southern 1975). The restriction fragments were detected with 14 (Ba1, Ba2, Ba5, B7, B10, B13, B18, B19, B20, B22, B25, B28, B29 and X6) of tobacco cp DNA probes (Sugiura et al. 1986), covering the entire chloroplast genome. The probes were chemiluminescently labeled and were visualized using ECL direct labeling and detection kits (Amersham) according to the protocols of the manufacturer. The temperatures of hybridization and washing were kept at 37° and 50°, respectively. For removal of the probe, a boiling solution of 0.5% Sodium Dodecyl Sulfate (SDS) was poured onto the membranes, which were rehybridized with other probes.

Phylogenetic analysis——The sizes of restriction fragments detected were calculated with *Hin*d III-digested lambda DNAs as standard. In cases where we used overlapping and adjacent probes, autoradiograms from the relevant probes were overlaid to determine whether one mutation was repeatedly counted or not. Thus, the data matrix was constructed as binary data based on the presence or absence of an interpretable restriction site.

Phylogenetic analysis was performed with PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2002). The restriction site gains and losses were equally weighted in all analyses. Most parsimonious trees were obtained using a heuristic search with 500 replicates of random addition sequence, tree bisection-reconnection (TBR) branch swapping algorithm and MULTREES.

Bootstrap (Felsenstein 1985) and decay analyses (Bremer 1988) were performed to determine the strength of support for monophyletic groups in the phylogenetic tree. The bootstrap analysis was conducted with 1000 replicates through heuristic search with 500 replicates of random addition sequence, TBR branch swapping algorithm and

MULTREES on. The decay analysis was heuristically carried out to examine the trees of one and two steps longer than the most parsimonous trees. Decay indices indicate the number of steps longer than the most parsimonous tree at which a given branch collapses.

Results

A total of 83 restriction site mutations were detected among 65 plants and 24 restriction enzymes. All site mutations are listed in Table 2. Consequently, the cpDNAs were classified into 29 different types (see Table 1). The site mutations result in associated fragment gains and losses without any changes in total size of such fragments. When the site mutations generate small restriction fragments that run off the gel, it is quite difficult to distinguish these site mutations from length mutations given simple changes of fragment sizes. In fact, we some-

Table 2. Restriction site mutations in cpDNA of the Far Eastern and European *Bupleurum* species. Probe numbers correspond to tobacco cpDNA clones (Sugiura et al. 1986). Type of cpDNA for each plant is shown in Table 1

No.	Enzyme	Probe	Fragments (kbp)	cpDNA type
1	*Apa* I	Ba2	11.2→7.0 + 4.2	26, 28, 29
2	*Apa* I	B7	10.2→6.2 + 4.0	28, 29
3	*Ase* I	B7	4.4→3.3 + 1.1	4
4	*Ase* I	B10	4.3 + 1.2→5.5	29
5	*Ase* I	B20	3.8→2.5 + 1.3	26
6	*Ava* I	Ba1	7.2 + 2.0→9.2	2, 3, 7, 11, 21, 22, 28, 29
7	*Ava* I	B5	3.5→3.1 + 0.4	25–29
8	*Ava* I	B5	3.1→1.7 + 1.4	27
9	*Ava* I	B7	4.3→3.3 + 1.0	2, 3, 7, 11, 21, 22
10	*Ava* I	B7	1.7 + 1.4→3.1	2
11	*Ava* I	B13	1.1 + 0.4→1.5	28, 29
12	*Ava* I	B18	1.7 + 1.1→2.8	25–29
13	*Ava* I	B19	5.1→3.4 + 1.7	29
14	*Ava* I	B19	6.8→4.8 + 2.0	6
15	*Ava* I	B22	5.0 + 3.3→8.3	7, 25, 28
16	*Bam* HI	Ba2	5.5→3.0 + 2.5	25–28
17	*Bam* HI	B13	3.8 + 1.2→5.0	25, 29
18	*Bam* HI	B20	8.3→4.8 + 3.5	29
19	*Bam* HI	B20	8.4 + 5.8→13.2	28
20	*Bam* HI	B25	3.0→1.7 + 1.3	25
21	*Bam* HI	B25	10.7→9.2 + 1.5	28, 29
22	*Bcl* I	B7	2.8 + 1.7→4.5	5, 6, 8, 15–19
23	*Bcl* I	B7	1.8→1.0 + 0.8	27
24	*Bcl* I	B13	2.8→1.8 + 1.0	26
25	*Bcl* I	B19	9.4→6.7 + 2.7	26
26	*Bcl* I	B20	4.6 + 1.8→6.4	27, 28
27	*Bcl* I	B25	7.7 + 3.3→11.0	27
28	*Bgl* I	Ba1	17.5→12.5 + 5.0	28, 29
29	*Bgl* I	B19	12.0→7.4 + 4.6	25
30	*Bgl* I	B20	14.4 + 8.9→23.3	28, 29
31	*Bgl* II	B7	2.9 + 1.6→4.5	22
32	*Csp* 45I	Ba1	4.4 + 1.3→5.7	25, 28, 29
33	*Csp* 45I	Ba2	4.7 + 3.4→8.1	5, 6, 8, 15, 17–19

Table 2. Continued

No.	Enzyme	Probe	Fragments (kbp)	cpDNA type
34	*Dra* I	Ba1	7.8→4.1 + 3.7	4, 25–27
35	*Dra* I	B7	3.3→2.4 + 0.9	26
36	*Eco* O109I	Ba1	7.9→5.2 + 2.7	28, 29
37	*Eco* O109I	Ba2	3.5→2.8 + 0.7	28, 29
38	*Eco* O109I	Ba2	4.8→2.7 + 2.1	27
39	*Eco* O109I	B20	3.4 + 3.1→6.5	28, 29
40	*Eco* RI	Ba1	4.9 + 1.1→6.0	3, 7, 21, 22, 27
41	*Eco* RI	B13	3.2→2.5 + 0.7	27
42	*Eco* RI	B20	8.2 + 4.4→12.6	6, 17, 18, 25, 26
43	*Eco* RV	Ba1	5.4 + 1.2→6.6	12
44	*Eco* RV	Ba1	5.4 + 4.6→10.0	28
45	*Eco* RV	B7	2.4 + 1.0→3.4	20, 25, 26, 28, 29
46	*Eco* RV	B7	1.3 + 1.0→2.3	27
47	*Eco* RV	B25	6.4→3.9 + 2.5	11
48	*Eco* RV	B25	7.6 + 6.4→14.0	12, 28, 29
49	*Eco* RV	B25	6.4 + 4.5→10.9	25, 26, 28
50	*Eco* T22I	B22	6.8 + 2.5→9.3	5
51	*Eco* T22I	B22	6.8→5.2 + 1.6	28, 29
52	*Eco* T22I	B25	19.0→14.6 + 4.4	29
53	*Eco* T38I	Ba1	3.3→1.8 + 1.5	28, 29
54	*Eco* T38I	Ba2	11.0→8.5 + 2.5	5, 6, 8, 15–19
55	*Eco* T38I	B10	6.0→4.2 + 1.8	26
56	*Eco* T38I	B13	6.0→4.3 + 1.7	26
57	*Eco* T38I	B20	2.3 + 1.9→4.2	26
58	*Eco* T38I	B29	1.3 + 1.0→2.3	27
59	*Hinc* II	Ba2	5.3→4.1 + 1.2	28, 29
60	*Hinc* II	X6	5.3→3.3 + 2.0	22
61	*Hinc* II	B18	1.6→1.0 + 0.6	25~29
62	*Hinc* II	B20	2.5→1.4 + 1.1	6
63	*Hinc* II	B22	7.0→4.5 + 2.5	28, 29
64	*Hinc* II	B22	7.0 + 1.8→8.8	21
65	*Hinc* II	B25	3.7→2.1 + 1.6	6, 24
66	*Hinc* II	B25	3.8 + 1.6→5.4	25
67	*Hinc* II	B25	8.4→5.7 + 2.7	14, 25, 28, 29
68	*Hind* III	Ba1	4.0→2.1 + 1.9	2, 3, 7, 11, 13, 14, 21, 22, 25–27
69	*Hind* III	Ba1	7.3 + 3.7→11.0	25–29
70	*Hind* III	Ba1	3.0 + 2.2→5.2	26
71	*Hind* III	Ba2	8.3 + 5.7→14.0	25, 28
72	*Hind* III	B19	3.7 + 3.2→6.9	28, 29
73	*Hind* III	B20	6.2 + 4.9→11.1	9, 10, 28, 29
74	*Hind* III	B25	2.4→1.5 + 0.9	26
75	*Sac* I	B5	3.4→2.8 + 0.6	26
76	*Sca* I	Ba1	4.4→2.9 + 1.5	23
77	*Sca* I	B7	2.0 + 1.0→3.0	5, 8, 15, 17, 18, 26
78	*Xba* I	X6	4.0 + 1.2→5.2	26
79	*Xba* I	B7	6.6 + 1.7→8.3	18, 28, 29
80	*Xba* I	B19	8.2→4.5 + 3.7	26
81	*Xba* I	B22	3.4→2.5 + 0.9	26
82	*Xba* I	B25	10.1→6.2 + 3.9	8, 9
83	*Xho* I	B25	11.5→8.0 + 3.5	25

times observed such cases. Also, we detected putative length mutations which were defined by changes of fragment sizes large enough to detect, but were nevertheless undetected. These cases were treated as length mutations not site mutations. In addition, we recognized very complex restriction site change patterns which were not easy to interpret. Hence, only the unambiguous site mutations (Table 2) were used in phylogenetic analysis. Figure 1 shows an example of the restriction site variation obtained from the combination of tobacco cpDNA probe #B18 and restriction enzyme *Ava* I. The European plants have a clear restriction fragment of approximately 2.8 kbp, which is divided into two fragments of 1.7 kbp and 1.1 kbp in the Far Eastern plants (mutation No. 12 in Table 2). The other bands are observed in all plants.

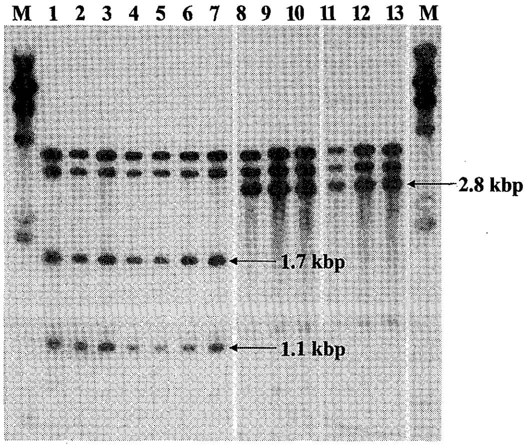

Fig. 1. Restriction site variation obtained from hybridization of tobacco cpDNA probe (#B18) to *Ava* I digested total DNAs of 13 *Bupleurum* plants. Arrows indicate the fragments showing 2.8 kbp = 1.7 + 1.1 (mutation number 12 in Table 2). Plant abbreviations are correspond to Table 1. Lane M: lambda *Hind* III digestion markers, lane 1: JP-F-Sh, lane 2: JP-F-Kol, lane 3: CH-C-Wal, lane 4: CH-C-Ch, lane 5: CH-S-Da, lane6: CH-S-Jul, lane 7: KO-F, lane 8: EU-F-Ne, lane 9: EU-F-Na, lane 10: TI, lane 11: EU-R, lane 12: EU-S, lane 13: EU-Fr.

We used site mutation data of 29 cpDNA types in the phylogenetic analysis. This approach treats all the plants with an identical cpDNA type as a single operational taxonomic unit. Phylogenetic analysis resulted in 36 most parsimonious trees of 107 steps with consistency index (Kluge and Farris 1969) of 0.776 and retention index (Farris 1989) of 0.795. Autapomorphies were included in all calculations. We constructed a strict consensus tree of these 36 most parsimonious trees with bootstrap values and decay indices (Fig. 2).

Our phylogenetic tree demonstrated the monophyly of the six subgroups in the Far Eastern plants of *Bupleurum falcatum* L. sensu lato and *B. longiradiatum* Turcz. (bootstrap value 79%, decay index >= 3), excluding European *B. falcatum* L. sensu stricto. Within this monophyletic group, we recognized three clades, in each of which Chinese individuals of *B. chinense* DC. were polyphyletically included.

All Japanese *B. falcatum* s. l. with 2n = 26 and 32 were contained in the first clade (clade I) with a relatively high degree of confidence (bootstrap value 85%, decay index 2). In addition, all members of Chinese *B. falcatum* s. l. with 2n = 26 and Chinese *B. komarovianum* Lincz. were included in this clade. Japanese plants with 2n = 26 and 32 had either type 2 or 3 cpDNA, and *B. komarovianum* also possessed type 3 (Table 1). Chinese plants with 2n = 26 showed the unique types 21 and 22, along with type 2 and 3.

Apart from the Japanese clade, all members of Chinese *B. scorzonerifolium* Willd. formed the second clade (clade II: bootstrap value 78%, decay index 2). *Bupleurum scorzonerifolium* had five cpDNA types (types 15 to 19) which were not detected in any of the other plants.

The last clade (clade III) contained only two plants of Chinese *B. chinense* with low

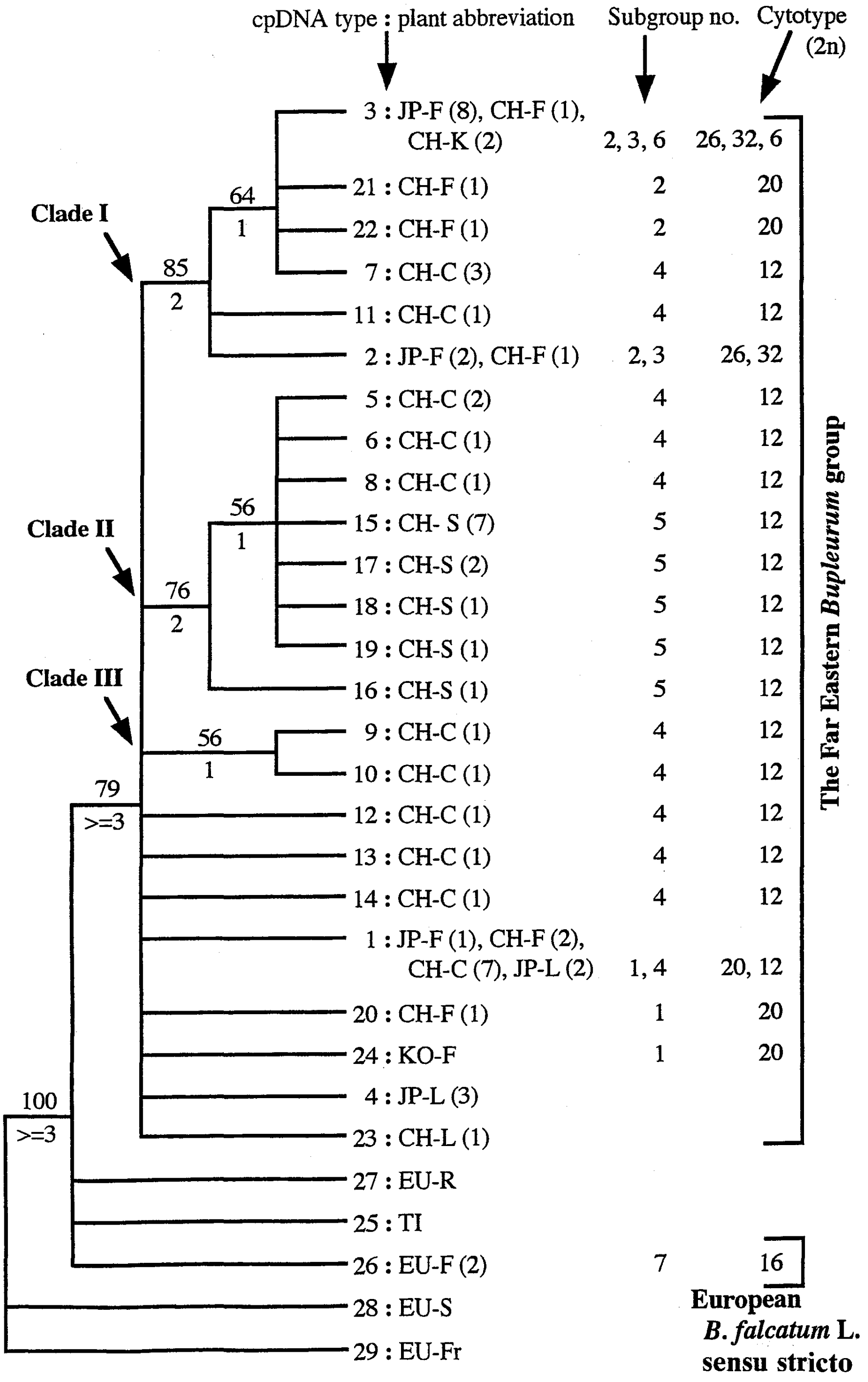

Fig. 2. Strict consensus tree of 36 most parsimonious trees from cpDNA restriction site variations of the Far Eastern and European *Bupleurum* (length = 107, CI = 0.776, RI = 0.795). Numbers above and below internodes indicate the bootstrap value from 1,000 replicates and decay index, respectively. Subgroup numbers, plant abbreviations and cpDNA types are correspond to Table 1. Numbers in parentheses indicate the number of accessious included.
]

support (bootstrap value 56%, decay index 1).

Bupluerum falcatum s. l. with 2n = 20 in Japan, China and Korea, and other Far Eastern plants did not form any clusters. However, the following 12 plants had an identical cpDNA type (type 1): one Japanese *B. falcatum* s. l. with 2n = 20 (JP-F-Na), two Chinese *B. falcatum* s. l. with 2n = 20 (CH-F-Da and CH-F-Do), seven Chinese *B. chinense* (CH-C-Li, CH-C-She1–4 and CH-C-Sha1–2) and two *B. longiradiatum* (JP-L-To, JP-L-Na).

European *B. falcatum* s. str. did not cluster with any other subgroups of the Far Eastern plants (Fig. 2). If we assume that European *B. falcatum* s. str. cluster with any other subgroups of the Far Eastern plants, the length of the phylogenetic tree needs to increase more than three steps than the most parsimonious tree of 107 steps. Furthermore, if we assume that European *B. falcatum* L. s. str. cluster with Japanese *B. falcatum* L. s. l. with 2n = 26 and 32, Chinese *B. falcatum* L. s. l. with 2n = 26, *B. komarovianum* and Chinese *B. scorzonerifolium*, the length of phylogenetic tree needs to increase more than five steps than the most parsimonious tree of 107 steps.

As for the relationships among sections, phylogenetic analysis strongly supported the monophyly of three sections, sect. Perfoliata (*B. rotundifolium*), sect. Longifolia (*B. longiradiatum*) and sect. Eubupleura (*B. falcatum* and *B. candollii*) (bootstrap value 100%, decay index >= 3). In this clade, sect. Longifolia (*B. longiradiatum*) and the Far Eastern sect. Eubupleura (*B. falcatum* s. l.) made a monophyletic clade, in which European sect. Eubupleura (*B. falcatum* s. str.) was not contained.

Discussion

In this study, the grouping in the Far Eastern *Bupleurum falcatum* sensu lato and European *B. falcatum* sensu stricto is consistent with their morphology and geographical distribution (Nakai 1937, Kitagawa 1947), and cytogenetics (Ohta 1986, 1991, unpublished), with exception of Chinese *B. chinense* and *B. falcatum* s. l. with 2n = 20. The traditional classification of *B. falcatum* is mainly based on its external morphology. Restriction site variation data produce more characters than external morphology alone. Therefore, the phylogentic relationships based on restriction site variation data are more reliable than the traditional classifications.

The phylogenetic tree supported the monophyly of six subgroups in the Far Eastern *B. falcatum* s. l., excluding European *B. falcatum* s. str. This result suggests that the Far Eastern *B. falcatum* s. l. is distinguished from European *B. falcatum* s. str., and that Japanese *B. falcatum* s. l. is more closely related than to the Far Eastern subgroups such as Chinese *B. scorzonerifolium* Willd., *B. chinense* DC. and *B. komarovianum* Lincz. to European *B. falcatum* s. str. This relationship is consistent with the taxonomical treatments of the Far Eastern plants by Nakai (1937), Kitagawa (1947) and Yamazaki (2001), recognizing that plants in Japan, China and Korea were distinct species from European *B. falcatum* s. str. Additionally, Ohta (1991) has reported that the European *B. falcatum* s. str. was quite different from the Far Eastern plants based on their karyology, as well as external morphology. From our results, the length of the most parsimonious tree needs to increase more than three steps if we assume that European *B. falcatum* s. str. cluster with the Far Eastern plants. Therefore, it is very difficult to accept that the Far Eastern plants are the same species as European *B. falcatum* s. str.

Within the Far Eastern group, Japanese *B. falcatum* s. l. with 2n = 26 and 32, Chinese *B. falcatum* s. l. with 2n = 26 and *B. komarovianum* made a single clade (clade I). In addition, Chinese *B. scorzonerifolium* formed another clade (clade II). These results agree with the taxonomical treatments of Japanese plants by Nakai (1937) and Kitagawa (1947). Nakai (1937) recognized the Japanese plants as a different variety from Chinese *B. scorzonerifolium*, and named them *B. scorzonerifolium* var. *stenophyllum*. Kitagawa (1947) recognized the Japanese plants as a different species from Chinese *B. scorzonerifolium*, and named them *B. stenophyllum*. Additionally, Chinese *B. scorzonerifolium* had a chromosome number of 2n = 12 (Gorovoy et al. 1980, Jiang et al. 1994, Pan et al. 1995), which was not observed in Japanese plants. Therefore, it is suggested that Japanese *B. falcatum* s. l. with 2n = 26 and 32 are closely related to Chinese *B. falcatum* s. l. with 2n = 26 and *B. komarovianum*, and are not included in the same taxon as Chinese *B. scorzonerifolium*. On the basis of cytogenetic and morphological study, and geographical distribution, Ohta (1998) has proposed to use *B. stenophyllum* for the Japanese plants with 2n = 26.

Japanese, Chinese and Korean *B. falcatum* s. l. with 2n = 20 did not form any clusters in the phylogenetic tree, indicating difference from Japanese *B. falcatum* s. l. with 2n = 26 and 32, and Chinese *B. falcatum* s. l. with 2n = 26, although a part of Japanese and Chinese *B. falcatum* s. l. with 2n = 20, and Chinese *B. chinense* had an identical cpDNA type (type 1). However, their phylogenetic relationships to other subgroups are still unclear. To clarify the relationships among subgroups in more detail, it is necessary to obtain more restriction site mutations by increasing the number of the restriction enzymes used or to analyze the informative gene sequences.

Chinese *B. chinese* is polyphyletic in our phylogenetic tree. This suggests a part of Chinese *B. chinese* is may be of hybrid origin with Chinese *B. scorzonerifolium*. To examine this possibility, we need to reconstruct the phylogeny of nuclear genes.

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ヨーロッパから中央アジア，東アジアを経て，日本まで広く分布するミシマサイコ *Bupleurum falcatum* L.（広義）の7つの亜グループの系統関係を明確にするため，葉緑体DNAの制限サイト変異による系統分析を行った．同属の5種の個体を加えた合計65個体を調査したところ，83個の変異が検出され，葉緑体DNAは29個のタイプに識別されることが判明した．最節約法により系統樹を構築した結果，極東アジアの *B. falcatum* の6亜グループは *B. longiradiatum* Turcz. と共に単系統群を形成し，この中にヨーロッパの *B. falcatum*（狭義）は含まれないことが示唆された．すなわち，極東アジアの広義 *B. falcatum* は系統学的にヨーロッパの狭義 *B. falcatum* と区別されることが分かった．また，日本の広義 *B. falcatum* のうち $2n = 26$ 及び32のサイトタイプは中国の広義 *B. falcatum* の $2n = 26$ のサイトタイプ及び *B. komarovianum* Lincz. と一緒にクレード（clade I）を形成していた．更に中国の *B. scorzonerifolium* Willd. はこのクレードとは別のクレード（clade II）を形成していた．一方，中国の *B. chinense* DC. はこれらの2つのクレードに含まれ，多系統的であった．つまり，日本の $2n = 26$ 及び32，及び中国の $2n = 26$ のサイト

タイプは中国の *B. scorzonerifolium* Willd. とは同一の分類群に含まれないことが示唆され，また日本，中国及び韓国の 2n = 20のサイトタイプとも異なることを示していた．

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